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Research Article

Functional analyses of neutrophil-like differentiated cell lines under a hyperglycemic condition

Tomoko Oya-Ito[1, 2], Hirotaka Naitou[1], Shuichi Masuda[1], Naohide Kinae[1, 2] and Norio Ohashi[1, 2]

[1] Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka, Japan
[2] COE program in the 21st Century and Global COE program, University of Shizuoka, Shizuoka, Japan

Diabetic patients are prone to severe bacterial infections. The functional alterations of neutrophils by hyperglycemia are thought to be partially responsible for such infections. In this study, we investigated the functional changes of neutrophil-like differentiated cell lines (dHL-60, dTHP-1, and dNB-4) by treatment with 5.5 mM, 11 mM, or 35 mM of glucose. In dHL-60 cells, the incubation with high glucose (35 mM) resulted in the enhancement of cell aggregation, the suppression of cellular fragility, the induction of reactive-oxygen species (ROS) production by phorbol myristate acetate (PMA) stimulation, and the impairment of phagocytosis. In dTHP-1 cells, the treatment with higher glucose generated the suppression of cellular fragility and extremely impaired phagocytosis (by 35 mM), and induced ROS production due to PMA stimulation (by 11 mM). Furthermore, the higher glucose exposure to dNB-4 cells enlarged intracellular vacuoles (by 35 mM) and induced ROS production due to PMA stimulation (by 11 mM). Since the ROS generation of those cells was enhanced only after PMA stimulation under the higher glucose conditions, glucose may have a priming effect rather than a triggering effect. These extraordinary sensitivities caused by the higher glucose treatments may reflect the dysfunction or overactivation of neutrophils.

Keywords: Diabetes / High glucose condition / Neutrophil-like differentiated human myeloid leukemia cell lines / Phagocytosis / Reactive oxygen species production

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1 Introduction

Severe bacterial infections are common in diabetic patients with hyperglycemia [1]. Neutrophils serve as a first defense line against pathogenic bacteria. The bactericidal activity of neutrophil is primarily due to oxidative pathway. By the assembly of enzymatic components (known as NADPH oxidase) segregated into the cytoplasm and plasma membrane of resting polymorphonuclear leukocyte (PMN) [2, 3], the bactericidal oxidants such as superoxide anion are produced from the activated neutrophils. Previous studies have shown that PMN in diabetic patients have impaired chemotaxis, phagocytosis, and oxidative and bactericidal activities [4 – 9]. A possible explanation for the impairment of PMN phagocytosis activities in diabetics is the influence due to the elevation of serum glucose concentration [10] and advanced glycation end-products (AGEs) [11, 12]. The short-term treatment (30 min) of PMN from healthy donors with high glucose concentration reduced the cellular respiratory burst [13]. Moreover, neutrophils from poorly controlled diabetics have impaired the ability of superoxide generation in the response to formyl-Met-Leu-Phe (fMLP) but not phorbol myristate acetate (PMA), and phospholipase D activity is decreased in the response to fMLP [10]. However, the detailed mechanisms of functional abnormalities of neutrophils under the hyperglycemic condition remain elusive. In our preliminary *ex vivo* experiment for 8 h or more, it was hard to examine the neutrophil function, because most neutrophils prepared from healthy humans rapidly died due to apoptosis.

Human promyelocytic leukemia cell lines, HL-60, THP-1, and NB-4, have been extensively used for several

Correspondence: Professor Norio Ohashi, 52-1 Yada, Shizuoka 422-8526, Japan
E-mail: ohashi@u-shizuoka-ken.ac.jp
Fax: +81-54-264-5793

Abbreviations: **AGEs**, advanced glycation end-products; **ATRA**, All-*trans* retinoic acid; **dHL-60**, differentiated HL-60; **dNB-4**, differentiated NB-4; **dTHP-1**, differentiated THP-1; **GM-CSF**, granulocyte-macrophage colony-stimulating factor; **NBT**, nitroblue tetrazolium; **PKC**, protein kinase C; **PMA**, phorbol myristate acetate; **PMN**, polymorphonuclear neutrophil; **ROS**, reactive-oxygen species

researches, *e. g*. the cellular differentiation and proliferation [14, 15], in worldwide laboratories. All-*trans* retinoic acid (ATRA) is well known as an inducer for cell differentiation, in particular it promotes the granulocytic maturation of human promyelocytic leukemia HL-60 cells [16 – 18]. The ATRA-differentiated HL-60 cells acquire the ability of reduction of nitroblue tetrazolium (NBT) [16 – 21], increase the tissue transglutaminase expression [19], modulate the proto-oncogene expression [20], induce the cell-surface expression of the Mac-i antigen [22], and up-regulate the protein kinase activity [21, 23]. NB-4 is a cell line derived from an acute promyelocytic leukemia patient and its differentiation can be induced by ATRA as well. Fleck *et al*. [24] have assessed the morphological changes such as cell spreading and flattening during the differentiation of HL-60 and NB-4 with ATRA toward a neutrophil-like phenotype by scanning electron microscopy. The cell-cycle progression of human monocytic cell line THP-1 is regulated by ATRA through the G1/S phase [25]. The mRNA expression of retinoic acid receptor (RAR) alpha of THP-1 cells is induced by granulocyte macrophage colony-stimulating factor (GM-CSF), and the NBT reducing activity of THP-1 cells is increased by the synergistic effect of ATRA and GM-CSF [26]. The expression of CD11b antigen (a cell surface marker of differentiation) on HL-60, NB-4 and THP-1 cells is increased by ATRA, as is phagocytosis activity [24, 25]. Thus, the HL-60 and NB-4 cells differentiated with ATRA, and the THP-1 cells differentiated with ATRA and GM-CSF are probably available for analysis of neutrophil-like function by treatment with high glucose for 24 h or more as hyperglycemic models. The aim of this study is to characterize the functional changes of those three differentiated cell lines under hyperglycemic conditions.

2 Materials and methods

2.1 Cell cultures

Three human leukemia cell lines (HL-60, NB-4 and THP-1) were maintained in RPMI 1640 medium including 11 mM glucose (a cell-maintaining medium, Iwaki, Japan) supplemented with 10% fetal calf bovine serum (FCS) at 37°C in 5% CO_2 atmosphere.

2.2 Cell differentiation

Cells were harvested during exponential growth and seeded at a density of 2×10^5 cells/ mL. The HL-60 and NB-4 cells were differentiated with 1 μM ATRA for 2 days in the cell-maintaining medium including 11 mM glucose (dHL-60 and dNB-4). For the differentiation of THP-1, the cells were cultured with 1 μM ATRA and 1 ng/mL GM-CSF for 2 days in the cell-maintaining medium (dTHP-1). The differentiation of those cells was monitored by NBT reducing assay as described later.

2.3 Treatment of cells with the different concentration of glucose

The differentiated or non-differentiated cells cultured in the cell-maintaining medium including 11 mM glucose were washed with PBS before experiments. For the mimicry of physiological conditions, the medium with addition of 5.5 mM glucose in glucose-free RPMI 1640 (Invitrogen, California, USA) was used. The cells (3×10^5 cells/mL of each) were incubated with a 5.5 mM glucose-containing medium (physiologic concentration), a 11 mM glucose-containing medium (*in vitro* cell-maintaining concentration), a 35 mM glucose-containing medium (addition of 24 mM glucose in cell-maintaining medium, high concentration), or a 24 mM mannitol-11 mM glucose-containing medium (addition of 24 mM mannitol in the cell-maintaining medium to rule out osmotic stress) for up to 4 days.

2.4 Cellular morphology, viability, vacuolization, and fragility

To characterize cellular morphology, the cells were prepared on glass slides by centrifugation using a Cytospin at $50 \times g$ for 2 min, and the slides were air-dried, fixed in methanol and stained with Diff-Quik (Dade Behring, Illinois, USA) and were observed at a magnification of $1000 \times$ under a light microscope. To examine cell viability, the number of living and dead cells in the cultures was determined by trypan blue dye exclusion under a phase contrast microscope. The ratio of living/dead cells was estimated as cell viability. For the observation of cellular vacuolization, the cells were stimulated with 10 ng/mL of PMA for 10 min, cytospined, fixed, and stained with Diff-Quik. The vacuolated cells were observed under a light microscope. For analysis of cellular fragility, the cells were stimulated with 10 ng/mL of PMA for 10 min, cytospined, fixed, and stained with Diff-Quik, and the number of cells was counted under a light microscope. The cells on the slide lack fragile cells, which were destroyed physically by cytospining. The percentage of cellular fragility was determined by the following equation: % of cellular fragility = [destroyed-Diff-Quik-stained cells/total-Diff-Quik-stained cells] $\times$ 100. Each individual experiment was repeated at least three times.

2.5 Cell aggregation assay

The number of aggregated cells and total cells was counted under a phase contrast microscope. The percentage of cells in aggregates was determined by the following equation: %

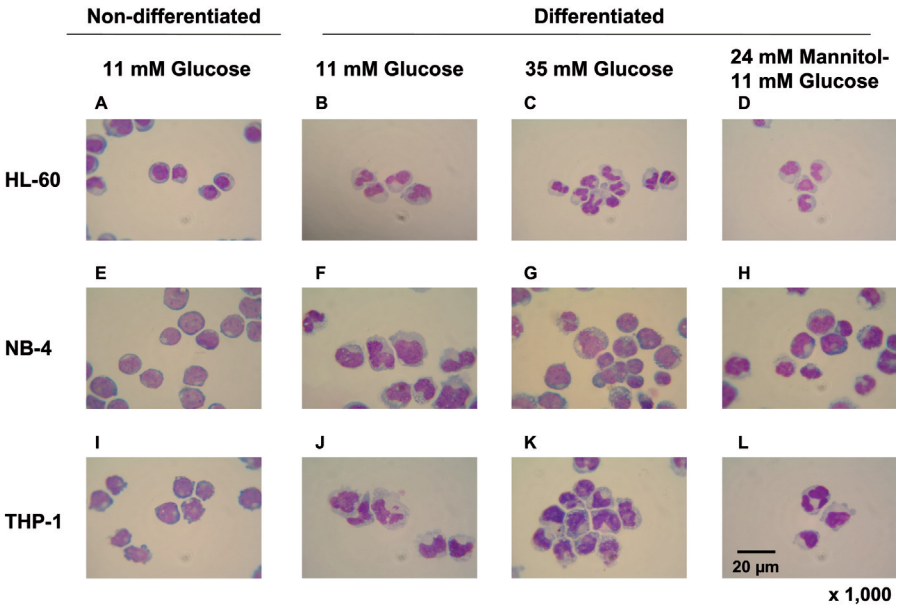

Figure 1. Morphological characteristics of non-differentiated and differentiated HL-60, NB-4, and THP-1 cells cultured with the different concentration of glucose. The cell lines were maintained in RPMI 1640 medium containing 11 mM glucose (A: HL-60, E: NB-4, I: THP-1). Differentiated cells were prepared by treatment with 1 μM ATRA for 2 days (dHL-60: B–D and dNB-4: F–H), or with 1 μM ATRA and 1 ng/mL GM-CSF for 2 days (dTHP-1: J–L). The differentiated cells were further cultured for 4 days with 11 mM glucose (B, F, and J), 35 mM glucose (C, G, and K), and 24 mM mannitol-11 mM glucose (D, H, and L). All cells used were stained with Diff-Quik.

of cells in aggregates = [aggregated cells/total cells] × 100. Each individual experiment was repeated at least three times.

2.6 NBT reducing assay

The cells ($0.4-1 \times 10^6$) were centrifuged at $700 \times g$ for 5 min, suspended with 1 mL PBS containing 2 mg/mL of NBT and 10 ng/mL of PMA, and incubated for 60 min at 37°C. The reaction was terminated by the addition of 0.4 mL of 2 N HCl and kept cooling on ice for 30 min. After centrifugation at $700 \times g$ for 5 min, the formazan deposits in the pellets were dissolved with 1 mL of DMSO, and the absorbance at 540 nm was measured. Each individual experiment was repeated at least four times. In some experiments, after NBT treatments with or without stimulation of PMA, the formazan-stained or non-stained cells were directly observed under a phase contrast microscope.

2.7 Phagocytosis of yeast by differentiated cells

Yeast particles (*Saccharomyces cerevisiae*; Sigma-Aldrich, Missouri, USA) were added to the suspension of differentiated cells in HBSS containing 5% fresh human serum and left in contact with the cells for 30 min at 37°C [27]. The final concentrations of yeast and differentiated cells were 6.25×10^7 particles/mL and 2.5×10^6 cells/mL, respectively. After centrifugation, the cell pellets were stained with Ziehl's carbolfuchsin solution (Sigma-Aldrich). The yeast particles outside the cells were stained red, and the yeast particles completely ingested by the cells were protected from taking up the stain. The cells containing unstained yeast particles were considered as cells capable of phagocytosis.

2.8 Statistical analysis

Data are presented as mean ± SD. Differences between mean values were determined by ANOVA. Differences with $p < 0.05$ were considered statistically significant.

3 Results

Three human myeloid leukemia cell lines, HL-60, NB-4, and THP-1, which were differentiated with ATRA alone or with ATRA and GM-CSF in the cell-maintaining medium containing 11 mM glucose for 2 days, showed PMN-like cell morphologies and the ratio of cytoplasm to nucleus was increased (Figs. 1A, B, E, F, I, and J). These differentiated cells, dHL-60, dNB-4, and dTHP-1, acquired NBT reducing abilities and their CD11b expressions were increased by FACS analysis (data not shown). By further treatment of the differentiated cells with 35 mM glucose or 24 mM manni-

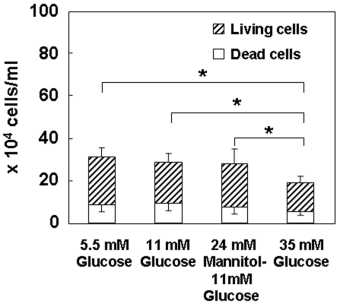

Figure 2. Effect of glucose on the cell concentration of dHL-60. After differentiation, the cells were exposed to the conditions of 5.5 mM glucose, 11 mM glucose, 35 mM glucose, and 24 mM mannitol-11 mM glucose for 4 days. The number of living and dead cells was determined by trypan blue dye exclusion under a phase contrast microscope and the sum is shown as the cell concentration (cells/mL). Data are mean ± SD from three or more independent experiments performed in quadruplicate. *, $p < 0.005$.

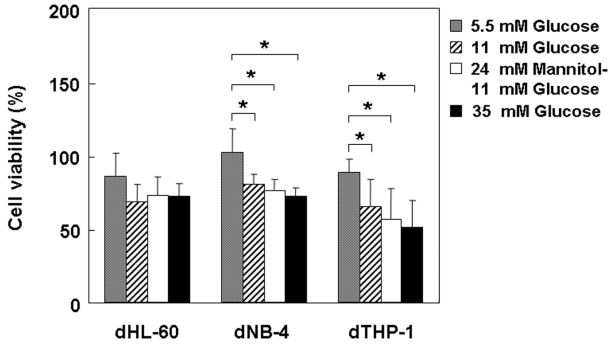

Figure 3. Effect of glucose on the cell viabilities of dHL-60, dNB-4, and dTHP-1. After differentiation, the cells were exposed to 5.5 mM glucose, 11 mM glucose, 35 mM glucose, and 24 mM mannitol-11 mM glucose for 4 days. The numbers of living and dead cells were determined by trypan blue exclusion under the phase contrast microscope. The ratio of living/dead cells was estimated as "cell viability". Data are mean ± SD from three or more independent experiments performed in quadruplicate. *, $p < 0.005$.

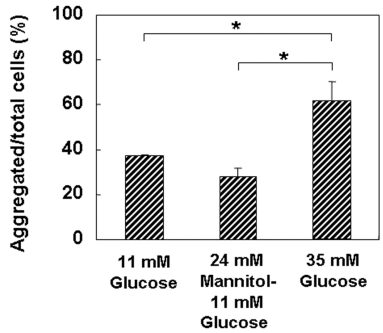

Figure 4. Effect of glucose on the cellular aggregation of dHL-60. The cells were exposed to 11 mM glucose, 35 mM glucose, and 24 mM mannitol-11 mM glucose for 4 days. The numbers of aggregated and total cells were counted under microscope and the percentage was determined. Data are mean ± SD from three to six replicate cultures. Each individual experiment was repeated a minimum of three times. *, $p < 0.05$.

tol-11 mM glucose for 4 days, there were little morphological differences between those glucose- and mannitol-treated cells (Figs. 1C, D, G, H, K, and L), and the cellular fragility under high glucose condition with 35 mM was reduced in the dHL-60 and dTHP-1 cells after PMA stimulation as described later.

In healthy human blood, the glucose concentration is in the range of 5 – 6 mM (*in vivo*), but the established cell lines such as HL-60, THP-1, and NB-4 were usually maintained with 11 mM glucose in RPMI 1640 medium (*in vitro*). Therefore, we first examined the effects of three different concentration of glucose, 5.5 mM (physiologic concentration), 11 mM (*in vitro* cell-maintaining concentration), and 35 mM (high concentration), on the differentiated cells. In the cell concentration of dHL-60 (Fig. 2), no significant difference was found among the differentiated cells treated with 5.5 mM glucose, 11 mM glucose, and 24 mM mannitol-11 mM glucose during the cultures for 4 days, whereas the cell concentration in dHL-60 treated with 35 mM glucose decreased approximately up to 65% of those in cells treated with the other concentration of glucose ($p < 0.005$). No effect of dNB-4 and dTHP-1 cells by treatment with 35 mM glucose for 4 days on the cell concentration was found (data not shown). Cell viabilities (a ratio of living/dead cells) of dNB-4 and dTHP-1 cultured for 4 days with 5.5 mM glucose were significantly increased when compared with those of the other concentrations of glucose (Fig. 3, $p < 0.05$), but there was no significant difference among the viability of dHL-60 cells treated with any different concentration of glucose. These results suggest that the culture for 4 days with higher glucose concentration (up to 35 mM) did not induce mortal damages such as cytotoxicity in those cell lines.

By treatment of dHL-60 with 35 mM glucose for 4 days, the cellular aggregation was increased (Fig. 4). The mean value of aggregation under the high glucose condition with 35 mM was estimated to be 62.1 ± 8.4%, while the corresponding values under the conditions with 11 mM glucose and 24 mM mannitol-11 mM glucose were 37.3 ± 0.3% and 27.8 ± 4.0%, respectively. Statistical analysis revealed that the high glucose condition with 35 mM significantly enhanced the cellular aggregation ($p < 0.05$). Further PMA stimulation did not affect the cell aggregation of dHL-60 cells (data not shown). The dNB-4 and dTHP-1 cells showed no differences in the cell aggregation among treatments with 35 mM glucose, 11 mM glucose, and 24 mM mannitol-11 mM glucose. However, additional PMA stimulation of dNB-4 and dTHP-1 cells treated with 35 mM glucose promoted the cellular aggregations (data not shown).

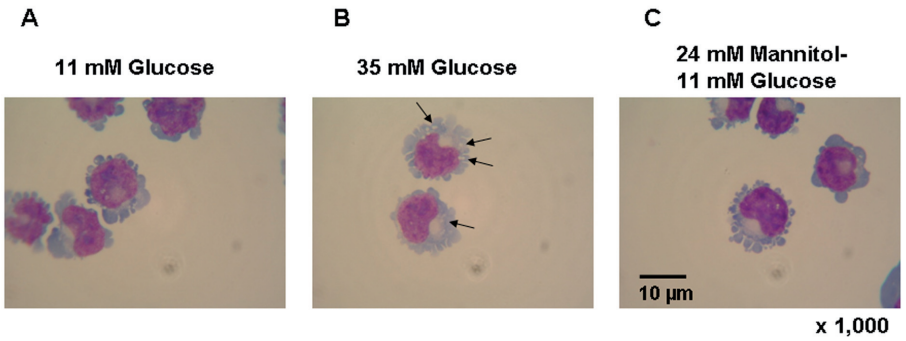

Figure 5. Effect of glucose on the intracellular vacuolation of dNB-4. The cells were exposed to 11 mM glucose (A), 35 mM glucose (B), and 24 mM mannitol-11 mM glucose (C) for 4 days. After stimulation with 10 ng/mL of PMA for 10 min, the exposed cells were stained with Diff-Quik. Arrows show cytoplasmic vacuoles.

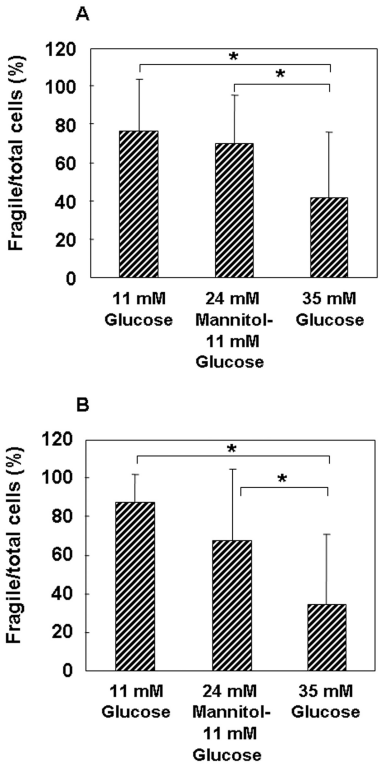

Figure 6. Effect of glucose on the cellular fragility of dHL-60 (A) and dTHP-1 (B). The cells were exposed to 11 mM glucose, 24 mM mannitol-11 mM glucose, and 35 mM glucose for 4 days. After stimulation with 10 ng/mL of PMA for 10 min, the exposed cells were stained with Diff-Quik and counted. The percentage of the cellular fragility was estimated by equation in Section 2. Data are mean ± SD from three to six replicate cultures. Each individual experiment was repeated a minimum of three times. *, $p < 0.02$.

By PMA stimulation after incubation with 35 mM glucose for 4 days (arrows in Fig. 5), the intracellular vacuolation of dNB-4 cells was induced. An equal amount of 24 mM mannitol-11 mM glucose did not enlarge vacuoles, suggesting that osmotic pressure did not affect such vacuolation. When the dNB-4 cells were not stimulated with PMA, no difference of vacuolation was observed among the cells treated with 35 mM glucose, 11 mM glucose, and 24 mM mannitol-11 mM glucose.

Since many fragile cells of dHL-60 and dTHP-1 were observed by PMA stimulation, the effect of glucose on the cellular fragility after PMA stimulation was examined. In dHL-60 and dTHP-1 cells, the fraction of fragile cells was reduced in the cultures with 35 mM glucose compared to the cultures with 11 mM glucose and 24 mM mannitol-11 mM glucose for 4 days (Fig. 6, $p < 0.02$). In both dHL-60 and dTHP-1 cells, the fragilities due to osmotic shock were not observed as shown in treatment with 24 mM mannitol-11 mM glucose. The suppressions of cellular fragility by the cultures with 35 mM glucose were observed only when the cells were triggered with PMA.

Production of reactive-oxygen species (ROS), especially superoxide anion, was determined by NBT reducing assay in cells cultured with different concentration of glucose. In all three differentiated cells cultured with 11 mM glucose for 24 h, the superoxide production by PMA stimulation were markedly increased compared to the culture with 5.5 mM glucose (Fig. 7A, $p < 0.0002$). In dHL-60 cultured with 35 mM glucose for 24 h, the superoxide production of the cells by PMA stimulation was1.5-fold increased compared to the cultures with 11 mM as well as 5.5 mM glucose (Fig. 7B, $p < 0.0001$). However, the short-term treatment for 5 h with 35 mM glucose did not affect the superoxide generation of dHL-60 (Fig. 7C). The superoxide generation of dNB-4 and dTHP-1 cells in culture with 35 mM glucose for 24 h, 11 mM glucose, and 24 mM mannitol-11 mM glucose was significantly increased compared with 5.5 mM glucose (Fig. 7B, $p < 0.0001$). The induction of superoxide production was not observed when these differentiated cells were cultured with concentrations of glucose without the PMA stimulation (Fig. 7A, and the microscopic observation of dHL-60 cells treated with 35 mM glucose as a representative in Fig. 7D).

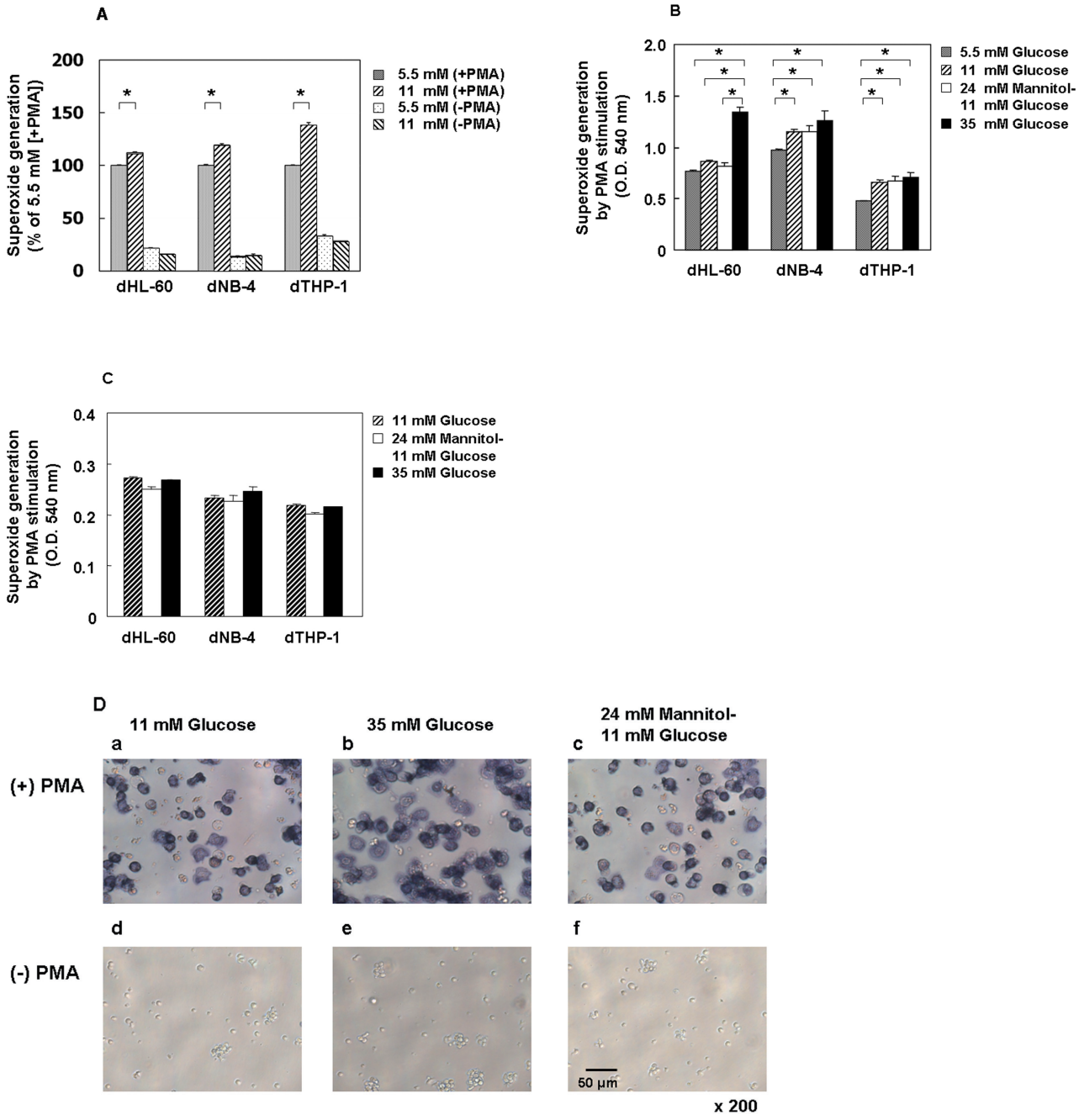

Figure 7. Effect of glucose on the superoxide generation of dHL-60, dNB-4, and dTHP-1 with or without PMA stimulation. The superoxide generation of differentiated and glucose-treated cells was evaluated by NBT reduction assay. Data are mean ± SD from three or more independent experiments. (A) Superoxide generation of the PMA-stimulated or non-stimulated cells after the culture for 24 h with 5.5 mM glucose (shaded bar) and 11 mM glucose (hatched bar). *, $p < 0.0002$. (B) Superoxide generation of the PMA-stimulated cells after the culture for 24 h with 5.5 mM glucose (shaded bar), 11 mM glucose (hatched bar), 24 mM mannitol-11 mM glucose (open bar), and 35 mM glucose (solid black bar). *, $p < 0.0001$. (C) Superoxide generation of the PMA-stimulated cells after the culture for 5 h (short-term) with 11 mM glucose, 24 mM mannitol-11 mM glucose, and 35 mM glucose, no significance was observed. (D) Morphological characteristics of the PMA-stimulated and non-stimulated dHL-60 cells cultured for 24 h with 11 mM glucose (a and d), 35 mM glucose (b and e), 24 mM mannitol-11 mM glucose (c and f). The blue-colored dHL-60 observed under the phase contrast microscope shows the formazan-deposited cells due to superoxide generation after PMA stimulation.

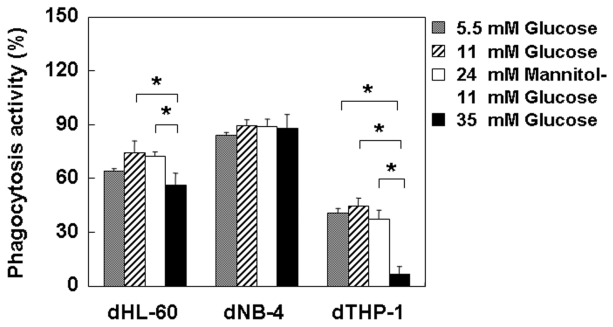

Figure 8. Effect of glucose on the phagocytosis activities of dHL-60, dNB-4, and dTHP-1. After differentiation, the cells were exposed to different concentrations of glucose (5.5 mM, 11 mM or 35 mM) for 24 h. Phagocytosis activity is expressed as the percentage of the cells ingesting. Data are mean ± SD from four independent experiments. *, $p < 0.001$.

The effect of glucose on the cellular phagocytosis was investigated. By culture with 35 mM glucose for 24 h, the phagocytosis of yeast particles by dHL-60 cells significantly decreased compared to the cultures with 11 mM glucose as well as 24 mM mannitol-11 mM glucose (Fig. 8, $p < 0.001$). In dTHP-1 cells incubated with 35 mM glucose for 24 h, phagocytosis activity extremely decreased compared to the cultures with the lower concentration of glucose and 24 mM mannitol-11 mM glucose ($p < 0.001$).

4 Discussion

To our knowledge, this study for the first time demonstrated that exposure of PMN-like differentiated cell lines to higher concentration of glucose (up to 35 mM) caused (i) the enhancement of cellular aggregation in dHL-60, (ii) the reduction of cellular fragility in dHL-60 and dTHP-1, (iii) the enlargements of intracellular vacuoles by PMA stimulation in dNB-4, (iv) the impairments of phagocytosis activities in dHL-60 and dTHP-1, and (v) the induction of superoxide generation by PMA stimulation. Because the culture of differentiated cell lines with 24 mM mannitol-11 mM glucose did not influence the aggregation, fragility or vacuolization, the osmotic effect can be excluded. The other studies showed that GM-CSF, which has a priming effect, stimulates neutrophil aggregation [28], but does not stimulate adherence to endothelial monolayers [29]. In contrast, tumor necrosis factor-α (TNF-α) and lipopolysaccharide, which are triggers, enhance neutrophil adherence to endothelium [30], but do not promote neutrophil aggregation [31]. Our study suggests that the expression of CD11b was not enhanced by high glucose treatment (data not shown). Vedder *et al*. [32] suggest that, as with adherence to endothelium, increased surface expression of CD11b is not the mechanism responsible for aggregation. Taken together, hyperglycemic conditions may play a role similar to GM-CSF in triggering immune responses of ATRA-induced differentiation.

The cytoplasmic vacuolization and/or toxic granulation of neutrophils have been demonstrated more often in bacteremic patients with high NBT reduction capacity [33]. Malcolm *et al*. [34] have reported that no significant differences in the extent of vacuolization were found among healthy donors, toxic patients without bacterial infections, and patients with bacterial infections in the absence of documented bacteremia. The extent of vacuolization was significantly greater in bacteremic patients when compared with the other patients, including patients with bacterial infection without bacteremia [34]. In our study, the effect of high glucose on the vacuolation of neutrophil-like cells by PMA stimulation to mimic bacterial infection was investigated. The dNB-4 cells under high glucose condition with 35 mM were extensively vacuolated by PMA stimulation, suggesting that the high glucose may enhance the ability of vacuolization due to some stimulation in neutrophil-like cells. The patients with type I diabetes mellitus were characterized by a significant impairment of the PMN-mediated phagocytosis [5, 35]. In our study, the phagocytosis activities of dHL-60 and dTHP-1 were significantly impaired in the cultures with high glucose for 24 h, suggesting the association with the pathogenicities in diabetes.

Oliveira *et al*. [36] have indicated that NBT reduction in incubation of rat pancreatic islets as well as neutrophils with 5.6, 8.3, and 16.7 mM of glucose for 1 h increased when compared with the absence of glucose. They further confirmed the involvement of NAD(P)H oxidase activation through protein kinase C (PKC) in the stimulatory effect of glucose by incubation with PMA (a PKC activator), bysindoylmaleimide (GF109203X) (a PKC-specific inhibitor), and diphenylene iodonium (an NAD(P)H oxidase inhibitor) to abolish the increase of NBT reduction induced by glucose. Although we do not know whether the NBT reduction in incubation of neutrophil-like cells with 5.5 mM glucose increases when compared with the absence of glucose, without PMA stimulation no superoxide production of differentiated cells was observed in incubation with any concentration of glucose in this study. Furthermore, the superoxide production by dHL-60, dNB-4, and dTHP-1 cells in incubation with higher glucose (11 or 35 mM) for long-term treatment (24 h) significantly increased when compared with 5.5 mM glucose (physiological concentration), in particular, the NBT reduction of dHL-60 in incubation with 35 mM glucose was extremely induced. However, no significant superoxide production in response to PMA was observed in the short-term treatment (5 h) of dHL-60 treated with 35 mM glucose, suggesting that longer-term treatment with high glucose seems to more enhance the induction of superoxide production. Since without PMA stimulation the NBT reduction of dHL-60 cells cultured with high glucose was not observed, the high glucose condi-

tion probably promotes the priming of neutrophil-like cells rather than the direct triggering.

Osmotic pressures of glucose-free RPMI, 5.5 mM glucose-containing RPMI, 11 mM glucose-containing RPMI, and 35 mM glucose-containing RPMI, which were estimated are approximately 250–290, 255–295, 265–300, and 300–350 mOsm/kg, respectively. In most of tissue culture media that are supplied by manufactures, the osmotic pressure is usually adjusted to the range of 260–320 mOsm/kg to keep isotonic condition. Osmotic pressures of 5.5 mM glucose- and 11 mM glucose-containing media are almost in the range. Only 35 mM glucose-containing medium may give the cell lines a little hypertonic shock. Therefore, we used 24 mM mannitol-11 mM glucose as such hypertonic control. In dHL-60 as shown in Fig. 7B, the ROS induction of 35 mM glucose (a little hypertonic)-treated cells was significantly increased by PMA stimulation when compared with those of the cells treated with 5.5 mM glucose (isotonic) and 11 mM glucose (isotonic), and 24 mM mannitol-11 mM glucose (the same osmotic condition as 35 mM glucose). This suggests that the increase of ROS production is dependent on very high glucose condition. In the cases of dNB-4 and dTHP-1, the ROS productions of 11 mM glucose (isotonic), 24 mM mannitol-11 mM glucose (a little hypertonic), and 35 mM glucose (a little hypertonic) were significantly increased by PMA stimulation when compared with 5.5 mM glucose (isotonic), suggesting that the increase of glucose concentration from 5.5 to 11 mM, but not hypertonic shock, seems to be related to the ROS induction. Thus, dHL-60 appears to become more sensitive by treatment with very high concentration of glucose such as 35 mM on ROS production by PMA stimulation, but dNB-4 and dTHP-1 are likely more sensitive by moderately high concentration (more than 5.5 mM and less than 35 mM) of glucose. The degree of effect may be dependent upon the sensitivity differences of those three cell lines against glucose concentration or treatment time, etc.

The reduction of NBT dye and the generation of ROS are thought to measure indirectly the bactericidal function of neutrophils. There is evidence demonstrating that bactericidal ROS production by activated PMN is reduced in diabetic patients with or without any infections [9] and in diabetic rats [37]. However, there is also evidence that it is normal [38] or increased [39] in patients with type 1 diabetes mellitus. Additionally, Nabi *et al*. and Bellinati-Peies *et al*. have suggested that reduction of NBT by neutrophils is not correlated with their bactericidal activity [35, 40]. They go on to suggest that only the complete absence of NBT reduction reflects low bactericidal activity in neutrophils. Based on those reports, a question is how we can explain exactly what the present findings of glucose-induced ROS production means in relation to bactericidal function of neutrophils in patients with type 1 diabetes. One of explanations is that the type 1 diabetes is much more complicated, because the patient blood with the diabetes included increased glycated proteins due to Maillard reaction, *e.g*. AGEs, as well as high glucose. Wong *et al*. [41] showed that AGEs stimulate an enhanced neutrophil respiratory burst mediated through the activation of cytosolic phospholipase A2 and generation of arachidonic acid. Moreover, De Toni *et al*. [42] have represented that in patients with diabetes the impact on PMN function is of multifactorial origin, and is probably correlated to the glucose level and to glycation of PMN protein, such as NADPH oxidase or myeloperoxidase. Alternatively, glucose in PMN may be reduced by aldose reductase to polyols, and this pathway requires NADPH, the coenzyme for the respiratory burst. They further found that superoxide production in response to opsonized zymosan was reduced in diabetic patients. The activation of protein tyrosine kinase is an important mechanism underlying transmembrane signaling and, the protein tyrosine phosphorylations, stimulated by zymosan receptor-mediated activation, might be caused by the activation of specific protein tyrosine kinase, whereas activation by PMA is probably mediated through another PKC type. In our study, therefore, we focused on the effect of high glucose alone (not including AGEs etc.) in ROS production at the mimicked initial step of maturated neutrophils released from bone marrow to blood using three differentiated cell lines.

Taken together, in response to glucose, HL-60 cells out of three cell lines seem to be better as a neutrophil model, but NB-4 and THP-1 (as well as HL-60) are still expected to have the potential responses similar to *in vivo* neutrophils under the consideration of sensitivity difference against glucose among those cell lines. By this possibility, we think that the three cell lines (HL-60, NB-4, and THP-1) are probably useful for better understanding of neutrophil function as a model *in vitro* experiment for long-term treatments (24 h or longer). Thus, this study provides significant and basic information regarding the effect of high glucose on the functional abnormalities of neutrophil-like differentiated cell lines.

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5 References

[1] Joshi, N., Caputo, G. M., Weitekamp, M. R., Karchmer, A. W., Infections in patients with diabetes mellitus. *N. Engl. J. Med.* 1999, *341*, 1906–1912.

[2] DeLeo, F. R., Quinn, M. T., Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. *J. Leukoc. Biol.* 1996, *60*, 677 – 691.

[3] Heyworth, P. G., Curnutte, J. T., Nauseef, W. M., Volpp, B. D., *et al.*, Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. *J. Clin. Invest.* 1991, *87*, 352 – 356.

[4] Shah, S. V., Wallin, J. D., Eilen, S. D., Chemiluminescence and superoxide anion production by leukocytes from diabetic patients. *J. Clin. Endocrinol. Metab.* 1983, *57*, 402 – 409.

[5] Marhoffer, W., Stein, M., Maeser, E., Federlin, K., Impairment of polymorphonuclear leukocyte function and metabolic control of diabetes. *Diabetes Care* 1992, *15*, 256 – 260.

[6] Marhoffer, W., Stein, M., Schleinkofer, L., Federlin, K., Evidence of *ex vivo* and *in vitro* impaired neutrophil oxidative burst and phagocytic capacity in type 1 diabetes mellitus. *Diabetes Res. Clin. Pract.* 1993, *19*, 183 – 188.

[7] Gallacher, S. J., Thomson, G., Fraser, W. D., Fisher, B. M., *et al.*, Neutrophil bactericidal function in diabetes mellitus: evidence for association with blood glucose control. *Diabet. Med.* 1995, *12*, 916 – 920.

[8] Repine, J. E., Clawson, C. C., Goetz, F. C., Bactericidal function of neutrophils from patients with acute bacterial infections and from diabetics. *J. Infect. Dis.* 1980, *142*, 869 – 875.

[9] Inoue, S., Lan, Y., Muran, J., Tsuji, M., Reduced hydrogen peroxide production in neutrophils from patients with diabetes. *Diabetes Res. Clin. Pract.* 1996, *33*, 119 – 127.

[10] Ortmeyer, J., Mohsenin, V., Inhibition of phospholipase D and superoxide generation by glucose in diabetic neutrophils. *Life Sci.* 1996, *59*, 255 – 262.

[11] Bernheim, J., Rashid, G., Gavrieli, R., Korzets, Z., Wolach, B., *In vitro* effect of advanced glycation end-products on human polymorphonuclear superoxide production. *Eur. J. Clin. Invest.* 2001, *31*, 1064 – 1069.

[12] Collison, K. S., Parhar, R. S., Saleh, S. S., Meyer, B. F., *et al.*, RAGE-mediated neutrophil dysfunction is evoked by advanced glycation end products (AGEs). *J. Leukoc. Biol.* 2002, *71*, 433 – 444.

[13] Nielson, C. P., Hindson, D. A., Inhibition of polymorphonuclear leukocyte respiratory burst by elevated glucose concentrations in vitro. *Diabetes* 1989, *38*, 1031 – 1035.

[14] Collins, S. J., The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood* 1987, *70*, 1233 – 1244.

[15] Janick-Buckner, D., Barua, A. B., Olson, J. A., Induction of HL-60 cell differentiation by water-soluble and nitrogen-containing conjugates of retinoic acid and retinol. *FASEB J.* 1991, *5*, 320 – 325.

[16] Breitman, T. R., Selonick, S. E., Collins, S. J., Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. USA* 1980, *77*, 2936 – 2940.

[17] Gallup, J. M., Barua, A. B., Furr, H. C., Olson, J. A., Effects of retinoid beta-glucuronides and N-retinoyl amines on the differentiation of HL-60 cells in vitro. *Proc. Soc. Exp. Biol. Med.* 1987, *186*, 269 – 274.

[18] Zile, M. H., Cullum, M. E., Simpson, R. U., Barua, A. B., Swartz, D. A., Induction of differentiation of human promyelocytic leukemia cell line HL-60 by retinoyl glucuronide, a biologically active metabolite of vitamin A. *Proc. Natl. Acad. Sci. USA* 1987, *84*, 2208 – 2212.

[19] Davies, P. J., Murtaugh, M. P., Moore, W. T., Jr., Johnson, G. S., Lucas, D., Retinoic acid-induced expression of tissue transglutaminase in human promyelocytic leukemia (HL-60) cells. *J. Biol. Chem.* 1985, *260*, 5166 – 5174.

[20] Imaizumi, M., Breitman, T. R., Changes in c-myc, c-fms, and N-ras proto-oncogene expression associated with retinoic acid-induced monocytic differentiation of human leukemia HL60/MRI cells. *Cancer Res.* 1988, *48*, 6733 – 6738.

[21] Fontana, J. A., Reppucci, A., Durham, J. P., Miranda, D., Correlation between the induction of leukemic cell differentiation by various retinoids and modulation of protein kinases. *Cancer Res.* 1986, *46*, 2468 – 2473.

[22] Koay, D. C., Sartorelli, A. C., Functional differentiation signals mediated by distinct regions of the cytoplasmic domain of the granulocyte colony-stimulating factor receptor. *Blood* 1999, *93*, 3774 – 3784.

[23] Wu, X. Z., Shao, G. Y., Chen, S., Wang, X. W., Wang, Z. Y., Studies on the relationship between protein kinase C and differentiation of human promyelocytic leukemia cells induced by retinoic acid. *Leuk. Res.* 1989, *13*, 869 – 874.

[24] Fleck, R. A., Athwal, H., Bygraves, J. A., Hockley, D. J., *et al.*, Optimization of nb-4 and hl-60 differentiation for use in opsonophagocytosis assays. *In Vitro Cell Dev. Biol. Anim.* 2003, *39*, 235 – 242.

[25] Chen, Q., Catharine Ross, A., Retinoic acid regulates cell cycle progression and cell differentiation in human monocytic THP-1 cells. *Exp. Cell Res.* 2004, *297*, 68 – 81.

[26] Shimizu, T., Takeda, K., Induction of retinoic acid receptor-alpha by granulocyte macrophage colony-stimulating factor in human myeloid leukemia cell lines. *Cancer Res.* 2000, *60*, 4544 – 4549.

[27] Kitagawa, S., Ohta, M., Nojiri, H., Kakinuma, K., *et al.*, Functional maturation of membrane potential changes and superoxide-producing capacity during differentiation of human granulocytes. *J. Clin. Invest.* 1984, *73*, 1062 – 1071.

[28] Arnaout, M. A., Hakim, R. M., Todd, R. F., 3rd, Dana, N., Colten, H. R., Increased expression of an adhesion-promoting surface glycoprotein in the granulocytopenia of hemodialysis. *N. Engl. J. Med.* 1985, *312*, 457 – 462.

[29] Lopez, A. F., Williamson, D. J., Gamble, J. R., Begley, C. G., *et al.*, Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor expression, and survival. *J. Clin. Invest.* 1986, *78*, 1220 – 1228.

[30] Gamble, J. R., Harlan, J. M., Klebanoff, S. J., Vadas, M. A., Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proc. Natl. Acad. Sci. USA* 1985, *82*, 8667 – 8671.

[31] Dahinden, C., Fehr, J., Granulocyte activation by endotoxin. II. Role of granulocyte adherence, aggregation, and effect of cytochalasin B, and comparison with formylated chemotactic peptide-induced stimulation. *J. Immunol.* 1983, *130*, 863 – 868.

[32] Vedder, N. B., Harlan, J. M., Increased surface expression of CD11b/CD18 (Mac-1) is not required for stimulated neutrophil adherence to cultured endothelium. *J. Clin. Invest.* 1988, *81*, 676 – 682.

[33] Hellum, K. B., Solberg, C. O., Granulocyte function in bacterial infections in man. *Acta Pathol. Microbiol. Scand. [C]* 1977, *85*, 1 – 9.

[34] Malcolm, I. D., Flegel, K. M., Katz, M., Vacuolization of the neutrophil in bacteremia. *Arch. Intern. Med.* 1979, *139*, 675 – 676.

[35] Nabi, A. H., Islam, L. N., Rahman, M. M., Biswas, K. B., Polymorphonuclear neutrophil dysfunctions in streptozotocin-induced type 1 diabetic rats. *J. Biochem. Mol. Biol.* 2005, *38*, 661 – 667.

[36] Oliveira, H. R., Verlengia, R., Carvalho, C. R., Britto, L. R., *et al.*, Pancreatic beta-cells express phagocyte-like NAD(P)H oxidase. *Diabetes* 2003, *52*, 1457 – 1463.

[37] Sato, N., Shimizu, H., Shimomura, Y., Uehara, Y. *et al.*, Reduced ability of neutrophils to produce active oxygen species in streptozotocin-induced diabetic rats. *Exp. Clin. Endocrinol.* 1992, *99*, 31 – 33.

[38] Thomson, G. A., Fisher, B. M., Gemmell, C. G., MacCuish, A. C., Gallacher, S. J., Attenuated neutrophil respiratory burst following acute hypoglycaemia in diabetic patients and normal subjects. *Acta Diabetol.* 1997, *34*, 253 – 256.

[39] Llorente, L., De La Fuente, H., Richaud-Patin, Y., Alvarado-De La Barrera, C., *et al.*, Innate immune response mechanisms in non-insulin dependent diabetes mellitus patients assessed by flow cytoenzymology. *Immunol. Lett.* 2000, *74*, 239 – 244.

[40] Bellinati – Pires, R., Carneiro-Sampaio, M. M., Colletto, G. M., Functional evaluation of human neutrophils. Is the bactericidal activity correlated with nitroblue tetrazolium reduction? *J. Investig. Allergol. Clin. Immunol.* 1992, *2*, 146 – 153.

[41] Wong, R. K. M., Pettit, A. I., Quinn, P. A., Jennings, S. C., *et al.*, Advanced glycation end products stimulate an enhanced neutrophil respiratory burst mediated through the activation of cytosolic phospholipase A2 and generation of arachidonic Acid. *Circulation.* 2003, *108*, 1858 – 1864.

[42] De Toni, S., Piva, E., Lapolla, A., Fontana, G., *et al.*, Respiratory burst of neutrophils in diabetic patients with periodontal disease. *Ann. NY Acad. Sci.* 1997, *832*, 363 – 367.

